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Postradiation Matrix Metalloproteinase-20 Expression and Its Impact on Dental Micromorphology and Radiation-Related Caries

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Keywords

Caries · Dental tissues · Dentin-enamel junction · Head-and-neck cancer · Matrix metalloproteinase-20 · Radiotherapy

Abstract

Recent evidence suggests that head-and-neck radiotherapy (HNRT) increases active forms of matrix metalloproteinase-20 (MMP-20) in human tooth crowns, degrading the dentin-enamel junction (DEJ) and leading to enamel delamination, which is a pivotal step in the formation of radiation-related caries (RRC). Additional participation of enzymatic degradation of organic matrix components in caries progression was attributed to MMP-20 in dentin. Therefore, the current study tested the hypothesis that MMP-20 is overexpressed in the DEJ, dentin-pulp complex components, and carious dentin of post-HNRT patients, leading to detectable

micromorphological changes to the enamel and dentin. Thirty-six teeth were studied, including 19 post-HNRT specimens and 17 nonirradiated controls. Optical light microscopy was used to investigate the micromorphological components of the DEJ, dentin-pulp complex components, and carious dentin. The samples were divided into 2 subgroups: nondemineralized ground sections ($n = 20$) and demineralized histological sections ($n = 16$). In addition, immunohistochemical analysis using the immunoperoxidase technique was conducted to semiquantitatively assess MMP-20 expression in the DEJ, dentin-pulp complex components, and carious dentin. No apparent damage to the DEJ microstructure or other dentin-pulp complex components was observed and no statistically significant differences were detected in MMP-20 expression ($p > 0.05$) between the irradiated and control groups. This study rejected the hypothesis that MMP-20 is overexpressed in the DEJ, dentin-pulp complex components, and carious dentin of post-HNRT patients,

leading to detectable micromorphological changes. Hence, direct effects of radiation may not be regarded as an independent factor to explain aggressive clinical patterns of RRC.

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Head-and-neck cancer (HNC) represents 6% of all malignancies, and approximately 670,000 new cases are diagnosed annually worldwide [Argiris et al., 2008]. Treatment usually involves surgery, chemotherapy, and radiotherapy (RT) (head-and-neck radiotherapy; HNRT) with high doses of radiation, alone or in combination with chemotherapy [Matzinger et al., 2009]. Though effective in cancer treatment, HNRT has a negative impact on the solid tissues surrounding the tumor within the radiation field, and consequently, on the patient's quality of life [Sciubba and Goldenberg, 2006].

The direct impact of radiation on the dental tissues of cancer patients remains unclear and a matter of intense academic debate. Although many studies have suggested direct radiogenic damage to teeth, leading to radiation-related caries (RRC) [Pioch et al., 1992; Grötz et al., 1997], others have linked the elevated risk of caries in post-HNRT patients to the indirect effects of RT in the head-and-neck region [Lieshout and Bots, 2014]. These include hyposalivation, oral microbiota alterations, impaired self-cleaning properties, poor oral hygiene prior to and after treatment, increased dietary intake of carbohydrates, and insufficient fluoride exposure [Kielbassa et al., 2006; Silva et al., 2009; Faria et al., 2014]. At least 28% of these patients have been estimated to present a higher risk of these aggressive caries, but the real number may be even higher [Hong et al., 2010].

The enamel organic matrix and the dentin-enamel junction (DEJ) act in synergy to preserve the adhesion between the enamel layer and the underlying dentin [McGuire et al., 2014b]. A recent study suggested that HNRT is able to increase the active forms of matrix metalloproteinase (MMP)-20 in irradiated tooth crowns [McGuire et al., 2014a]. MMP-20 (enamelysin) is considered a tooth-specific MMP [Llano et al., 1997] because, in addition to tooth tissues, it has been detected in vivo only in some odontogenic tumors [Turk et al., 2006]. MMP-20 degrades amelogenin, type IV and V collagens, aggrecan, fibronectin, laminin, and tenascin-C [Turk et al., 2006; Mazzoni et al., 2012]. It is essential for the proper junctional adherence of enamel to dentin and enamel formation, as MMP-20^{-/-} mice have shown an amelogenesis imperfect phenotype, wherein a thinner enamel layer that delaminates from dentin has been observed [Caterina et

al., 2002]. In irradiated teeth, MMP-20 activation has been suggested to degrade the DEJ and surrounding enamel and dentin organic matrix [McGuire et al., 2014a], eventually leading to enamel delamination, which is considered a fundamental clinical step in the onset and progression of RRC [Walker et al., 2008]. There has been additional interest in the role of the remaining dentin-bound and odontoblast-released MMP-20 since previous findings have indicated that this enzyme might participate in caries progression of mature human teeth [Sulkala et al., 2002].

In the present paper, a micromorphological study was combined with an immunohistochemical analysis of MMP-20 to evaluate hard and organic tissues of teeth extracted from HNC patients to determine whether radiation can directly alter these components. The main tested hypothesis was that MMP-20 would be overexpressed in the DEJ of in vivo irradiated teeth, leading to micromorphological changes in the DEJ and, consequently, enamel delamination. Secondly, we hypothesized that other dentin-pulp complex tissues may also express higher levels of MMP-20 in response to radiation, leading to micromorphological changes in the dentin, odontoblasts, and dental pulp, thereby facilitating dentin demineralization and explaining the notorious aggressive patterns of the progression of RRC throughout the dentin and adjacent dental tissue components.

Materials and Methods

Patients and Specimen Collection

This study was approved by the Ethics Committee of the Piracicaba Dental School (protocol 012/2013), University of Campinas, Piracicaba, Brazil, and it was conducted in accordance with the Declaration of Helsinki. Noncarious and carious erupted teeth ($n = 36$) from HNC patients were collected following the protocol of the service of origin and independently of the particulars of the present study. Dental extractions were performed due to caries or advanced periodontal disease in both tooth groups (irradiated and nonirradiated).

Teeth forming the irradiated group were extracted from patients subjected to clinical radiation protocols with tridimensional conformal HNRT in 6-mV linear accelerators on the Synergy Platform (Elekta AB, Stockholm, Sweden) with a cumulative dose that ranged from 40 to 70 Gy (2 Gy/day for a maximum of 5 days/week) 3–12 months after RT conclusion. All patients were diagnosed with squamous cell carcinomas, except for one who was diagnosed with non-Hodgkin lymphoma. Nonirradiated specimens were obtained from HNC patients before radiation treatment. The tridimensional HNRT plan of the patients was retrieved from the CMS system (XiO version 4.60; Elekta CMS, St. Louis, MS, USA) to study the radiation field and the total dose directed to the primary tumor and teeth. For clinical characterization of the patients in this

study, the electronic medical record system Tasy (Philips Clinical Informatics, Blumenau, Brazil) was consulted and data were collected. Information about age, gender, the primary tumor site, alcohol abuse and smoking habit, the tumor histological type, the clinical cancer stage (according the American Joint Committee on Cancer; AJJC), the total amount of radiation during treatment (Gy), the type of radiation plan, the extracted teeth, and the time between the end of HNRT and tooth extraction was retrieved from the patients' charts.

Immediately after the extractions, teeth were identified, placed in plastic containers with 10% buffered formalin solution, and fixed for at least 72 h at 4°C. The specimens were divided into 2 groups (irradiated and nonirradiated) and further divided into the following 2 subgroups according to histological preparation: subgroup 1 (nondemineralized samples) and subgroup 2 (demineralized samples).

Ground Section Preparation (Subgroup 1)

Twenty teeth, including irradiated ($n = 11$) and nonirradiated ($n = 9$) specimens were inspected and the dental calculus was removed with periodontal curettes. The samples were sectioned along their long axes with a diamond saw (Extex, Enfield, CT, USA) in a precision cutter (Buehler Isomet 1000; Lake Bluff, IL, USA), passing through the center of the deepest region of caries or dividing them into 2 equal halves to obtain a slice with a thickness of approximately 1.0 mm. The sections were then ground to a thickness of approximately 200 μm with silicone carbide sandpaper, following a sequence of 600, 1,200, 2,000, and 4,000 granulation. The final thickness was verified at the end of the process using a digital caliper (Standard Gage, Poughkeepsie, NY, USA).

Demineralization and Histological Preparation (Subgroup 2)

Sixteen teeth, including irradiated ($n = 8$) and nonirradiated ($n = 8$) teeth, were decalcified in Ana Morse's solution (equal volumes of 20% sodium citrate and 50% formic acid) at 4°C for 3 weeks, with changes every 2 days. The samples were embedded in Paraplast Plus® (Leica Biosystems Richmond, Inc., Richmond, IL, USA) to produce 5- μm sections on a microtome (Leica, Nussloch, Germany) in silanized slides for hematoxylin and eosin morphological evaluation and immunohistochemical analysis.

Optical Light Microscopy Analysis

For micromorphological study of the DEJ, enamel, and dentin components, an optical light microscope (Eclipse E200; Nikon, Tokyo, Japan) was used and one ground section of each specimen was analyzed. Tufts, lamellae, spindles, the type of DEJ (smooth or scalloped) in the different areas (cervical, medium, and incisal/occlusal thirds of the dental crown), striae of Retzius, and gnarled enamel were evaluated, as were interglobular dentin, incremental lines, Tome's granular layer, and tertiary dentin in the specimens of subgroup 1. In demineralized histological sections from specimens of subgroup 2, the DEJ and the dentin-pulp complex components were analyzed semiquantitatively. Finally, the patterns of caries were analyzed in the enamel and dentin following methods described previously [Silva et al., 2009].

Immunohistochemical Analysis

Histological sections of each demineralized specimen were deparaffinized in xylene. After deparaffinization, inhibition of endogenous peroxidase was performed by immersion in 10 volumes

of hydrogen peroxide (3 times for 5 min each). The sections were washed in 3 successive baths of phosphate-buffered saline (PBS; pH 7.4) for 5 min each. To better expose the epitopes, the histological sections were subjected to antigen retrieval with 0.5% trypsin for 1 h in a humid chamber at 37°C.

Nonspecific binding was blocked with bovine serum albumin (BSA: i.e., 3% BSA/PBS) for 30 min at room temperature. Each slide was then incubated with the primary anti-MMP-20 antibody (monoclonal antibody C7 for MMP-20; Fuji Chemical Industries, Toyama, Japan) diluted at 1:100 in PBS and an overnight incubation was carried out at 4°C in a humidifier. The sections were then washed with PBS in 3 baths of 5 min each and incubated with the biotinylated secondary antibody (LSAB-Link; DAKO Corporation, Carpinteria, CA, USA) at first and then washed with streptavidin/peroxidase system (biotin-labeled streptavidin; Dako Corporation, Carpinteria, CA, USA). The reaction was visualized with 3,3'-deaminobenzidine (DAB Substrate Kit®; Dako) applied for 90 s. The sections were counterstained using Mayer's hematoxylin and coverslips. Negative controls were performed with omission of the primary antibody, and human tooth germs were used as positive controls.

Statistical Analysis

Data was analyzed statistically using SAS software version 9.3 (SAS Institute Inc., Cary, N.C., USA) using the Cochran-Mantel-Haenszel test, with the significance level set at $\alpha = 0.05$.

Results

Patients and Specimens Features

The irradiated samples were obtained from a total of 19 post-HNRT patients (subgroup 1, $n = 11$; subgroup 2, $n = 8$). In subgroup 1 ten patients were male and 1 patient was female, and in subgroup 2 five patients were male and 3 were female. The mean age was 58 years (range 36–74) and 60 years (range 52–75) in subgroups 1 and 2, respectively. Smoking habit, as well as alcohol abuse, was recorded in 9 and 6 patients, respectively. Tumors sites were as follows: tongue ($n = 3$), oropharynx ($n = 3$), larynx ($n = 2$), base of tongue ($n = 2$), and maxillary sinus ($n = 1$) in the first subgroup. In the former subgroup, tumors locations were: tongue ($n = 4$), soft palate ($n = 2$), and nasopharynx ($n = 1$), and 1 case represented an unknown primary tumor with a level II cervical metastatic lymph node. All cases in subgroup 1 represented stage IV of the disease, meanwhile 2 cases were stage III and 6 cases were stage IV in subgroup 2. Nine patients in subgroup 1 were treated with a chemoradiotherapy protocol and 2 were treated exclusively with RT. Similarly, 7 patients in subgroup 2 were treated with chemoradiotherapy and 1 was treated with RT alone. The mean (\pm SD) total dose of radiation delivered to the tumors was 68.6 ± 2.4 Gy in subgroup 1 and 66.25 ± 10.6 Gy in subgroup 2.

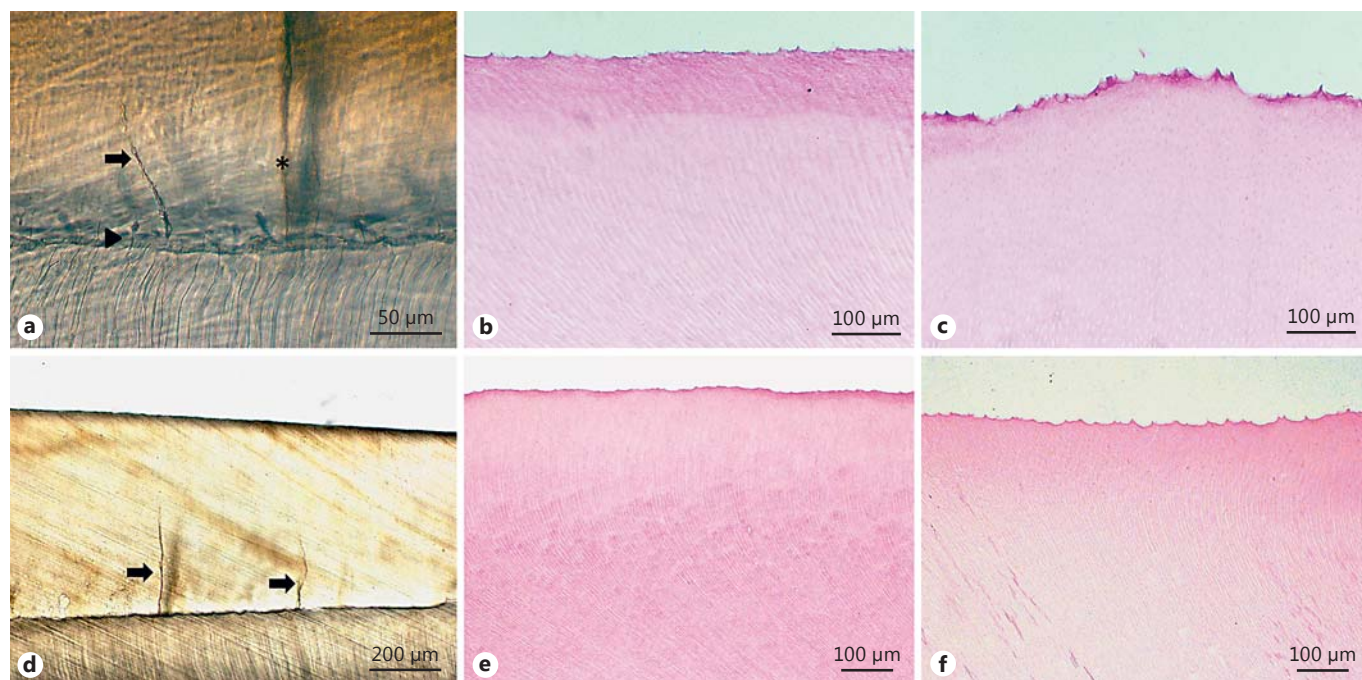


Fig. 1. Optical light micrographs of enamel and DEJ morphology of the irradiated (group 1; **a–c**) and nonirradiated (group 2; **d–f**) specimens. **a** Detail of preserved tufts (arrowhead), spindles (arrow), and lamellae (asterisk) in an irradiated specimen. Preservation of smooth (**b**) and gnarled (**c**) patterns of DEJ in both areas,

showing no disruption or changes. **d** Micromorphological components of enamel with clear visualization of spindles (arrows). Smooth (**b**) and gnarled (**c**) patterns of DEJ representing cervical and incisal areas, respectively.

Morphological Analysis

A total of 11 incisors, 9 canines, 7 premolars, 7 molars, and 2 undefined teeth were distributed in both groups. Caries was observed in 21 specimens (15 incisal/occlusal caries, 8 proximal caries, and 16 cervical caries). Fifteen specimens did not present any surface affected by caries. The characteristic brown discoloration of RRC-affected teeth was present in 10 out of 12 (83%) irradiated teeth, but only 1 out of 9 (11%) of nonirradiated teeth presented a similar pattern. Nine specimens presented a superficial filling.

Morphological analysis of the ground sections (group 1) of all post-HNRT specimens showed no significant difference in the micromorphological components of dental hard tissues, including enamel and dentin, between the irradiated and nonirradiated groups.

There was a trend of dominance of the conventional scalloped DEJ pattern in irradiated teeth compared to nonirradiated teeth (10 out of 11 vs. 5 out of 9, respectively; $p = 0.07$). Middle and incisal/occlusal areas had a scalloped DEJ pattern in all of the teeth, irrespectively of irradiation. None of the specimens showed gap formation,

cracking, or disruption of the DEJ. The presence of tufts, spindles, lamellae (Fig. 1a vs. d), striae of Rezius, and gnarled enamel did not differ between the irradiated and nonirradiated teeth. In dentin, interglobular dentin was encountered in 10 out of 11 (90.9%) irradiated teeth but only in 5 out of 9 (55.6%) nonirradiated teeth ($p = 0.07$). There were no differences between the presence of the incremental lines, Tome's granular layer, or tertiary dentin in response to caries between the irradiated and nonirradiated teeth. Also, patterns of demineralization in RRC showed half-moon-shaped lesions presenting softened dentin, superficially demineralized dentin, sclerotic dentin, and translucent zones, as would be seen in conventional caries. A summary of the micromorphological analysis of specimens from group 1 is presented in Table 1.

Histological Analysis

Histological components of the dentin-pulp complex of demineralized post-HNRT specimens (subgroup 2) were also apparently not changed by radiation and, again, the DEJ maintained its conserved pattern (Fig. 1b, c, vs. e, f). The pulpal components (odontoblast cell layer, extra-

Table 1. Micromorphological analysis of preserved DEJ, enamel, and dentin components of the nondemineralized specimens (subgroup 1)

	Irradiated	Nonirradiated	<i>p</i>
DEJ			
Cervical	10/11 SC (91) and 1/11 SM (9)	5/9 SC (57) and 4/9 SM (44)	0.07
Middle	11/11 SC (100) and 0/0 SM (0)	9/9 SC (100) and 0/0 SM (0)	na
I/O	9/9 SC (100) and 0/0 SM (0)	8/8 SC (100) and 0/0 SM (0)	na
Tufts			
Cervical	7/9 (78)	8/8 (100)	0.16
Middle	9/11 (89)	9/9 (100)	0.18
I/O	10/11 (91)	8/9 (90)	0.88
Spindles			
Cervical	8/9 (89)	5/8 (62)	0.21
Middle	8/11 (73)	8/9 (89)	0.38
I/O	9/9 (100)	8/9 (89)	0.31
Lamella			
Cervical	8/9 (89)	8/8 (100)	0.34
Middle	7/11 (64)	7/9 (78)	0.50
I/O	7/10 (70)	8/9 (89)	0.32
Enamel			
Striae of Retzius	5/10 (50)	7/9 (78)	0.22
Gnarled enamel	6/11 (54)	5/9 (55)	0.96
Dentin			
Interglobular dentin	10/11 (91)	5/9 (55)	0.07
Incremental lines	3/11 (27)	5/9 (55)	0.21
Tomes' granular layer	8/11 (73)	7/9 (78)	0.80
Tertiary dentin	3/11 (27)	3/9 (33)	0.77
Caries (I/O)	8/11 (73)	6/9 (67)	0.80
Caries (C)	8/11 (73)	7/9 (78)	0.77

Values in parentheses are percents. DEJ, dentin-enamel junction, SC, scalloped, SM, smooth, na, not available, I, incisal, O, occlusal, C, cervical.

Table 2. Micromorphological analysis of preserved DEJ and dentin-pulp complex components of the demineralized specimens (subgroup 2)

	Irradiated	Nonirradiated	<i>p</i>
DEJ	8/8 (100)	8/8 (100)	na
Dentin			
Cariou dentin	8/8 (100)	5/7 (71)	0.11
Tertiary dentin	3/8 (37)	3/7 (43)	0.77
Pulp			
Odontoblastic layer	5/5 (100)	4/6 (67)	0.17
Extracellular matrix and fibroblasts	3/5 (60)	3/5 (60)	1.00
Calcifications	3/8 (37)	3/7 (43)	0.77
Nerve bundles	5/6 (83)	6/7 (86)	0.77
Blood vessels	6/6 (100)	7/7 (100)	0.54

Values in parentheses are percents. DEJ, dentin-enamel junction; na, not available.

cellular matrix with fibroblasts, nerve bundles, blood vessels, and calcifications) did not differ in structure between the irradiated and nonirradiated groups. The pulp presented a polarized odontoblastic layer arranged in a palisade, subodontoblastic cell-poor layer of Weil and the central zone (rich in blood vessels, fibroblasts, and neural bundles) with preservation of the normal components and architecture (Fig. 2a vs. d). Tertiary dentine formation was present under the caries front (Fig. 2b vs. e). In the specimens presenting caries, an outer layer (caries-infected dentin) composed of disorganized dentin and bacterial colonies, as well as an inner layer with affected, but not disrupted, dentin was observed (Fig. 2c vs. f). No significant difference was encountered between irradiated and nonirradiated groups in any of the analyzed parameters including periodontal ligament (cementum and collagen fibers). The summary of the histological analysis of demineralized specimens (group 2) is presented in Table 2.

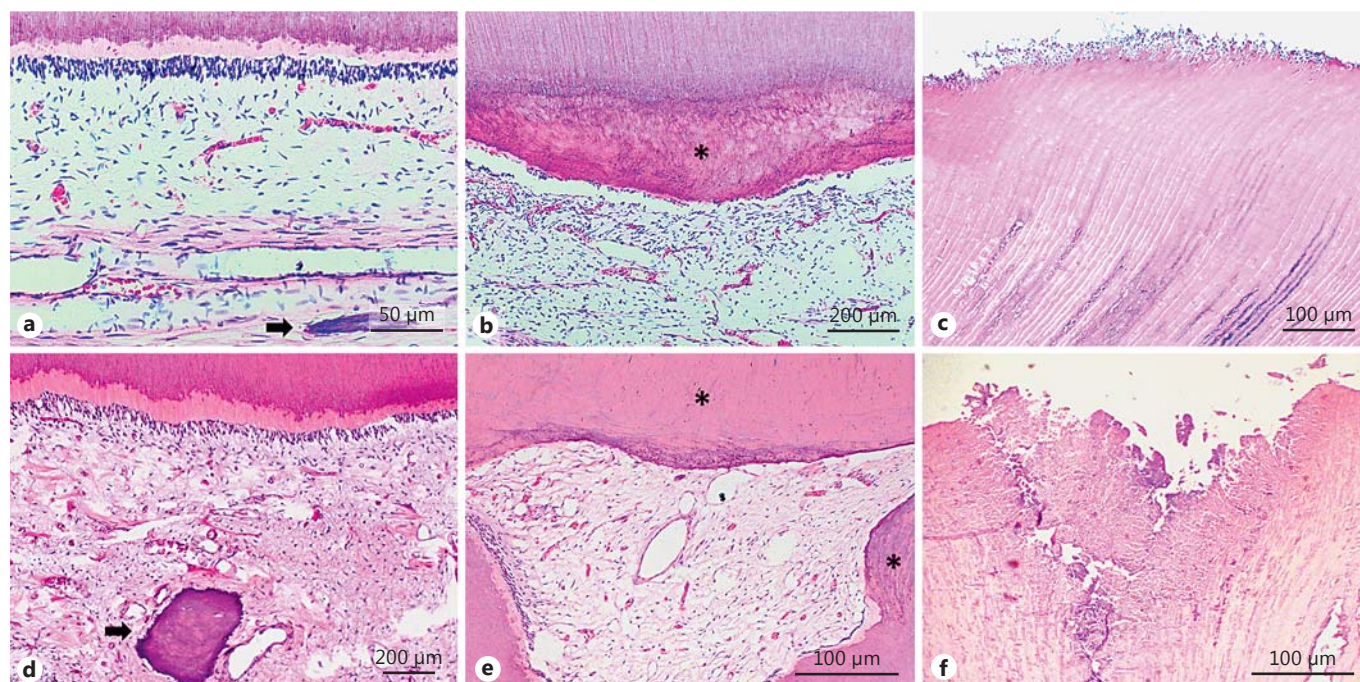


Fig. 2. Morphological analysis of dentin-pulp complex components and caries of the irradiated (group 1; **a–c**) and nonirradiated (group 2; **d–f**) specimens. **a** Preservation of the morphological dentin-pulp complex hierarchy in an irradiated sample. Arrow, dystrophic calcification. **b** Tertiary dentin formation with a poorly organized replacement odontoblast layer and mild pulp inflammation. Asterisk, tertiary dentin. **c** RRC showing an outer layer composed by disorganized

dentin and bacterial colonies, followed by an inner layer of affected dentin and dilated dentinal tubules underneath the demineralization front. **d** From the top: secondary dentin, predentin, odontoblast layer, cell-poor Weil zone, and neurovascular bundle in the inner region of the pulp with a dystrophic calcification (arrow). **e** Tertiary dentin formation. Asterisks, tertiary dentin. **f** Representation of an occlusal conventional caries showing a similar dentin breakdown.

MMP-20 Expression

MMP-20 expression was pronounced and intense along the DEJ of all of the irradiated and nonirradiated examined specimens (Fig. 3a vs. d). All of the teeth were also highly positive for carious dentine as well as sound dentine in some focal areas. The odontoblast cell layer was positive in 9 out of 11 teeth, showing in most of the cases a cytoplasmic dot pattern, though it was similar in both groups (Fig. 3b vs. e), while pre-dentin and pulp tissue demonstrated more variable staining. An intense staining was available within dilated dentinal tubules towards the pulp (Fig. 3c vs. f). No significant differences were detected between the irradiated and nonirradiated groups (Table 3).

Discussion

The results of the present study are in accordance with the evidence that teeth exposed to high cumulative radiation doses for HNC treatment present a particular risk for RRC [Walker et al., 2011]. Teeth that had been exposed

Table 3. Immunohistochemical analysis of MMP-20 in the demineralized specimens (subgroup 2)

	Irradiated	Nonirradiated	<i>p</i>
DEJ	8/8 (100)	8/8 (100)	na
Carious dentin	8/8 (100)	5/7 (71)	0.10
Sound dentin	8/8 (100)	8/8 (100)	na
Tertiary dentin	1/4 (25)	4/5 (80)	0.11
Predentin	3/6 (50)	4/7 (57)	0.80
Odontoblastic layer	5/5 (100)	4/6 (67)	0.17
Pulp (ECM)	3/5 (60)	3/5 (60)	1.00

Values in parentheses are percentages of positivity. DEJ, dentin-enamel junction; ECM, extracellular matrix; na, not available.

to in vivo irradiation were affected by caries that varied from early stages, characterized by brown discolorations affecting noncavitated enamel and incisal wear, to advanced cervical and incisal/occlusal cavitated lesions [Kielbassa et al., 2006; Silva et al., 2009].

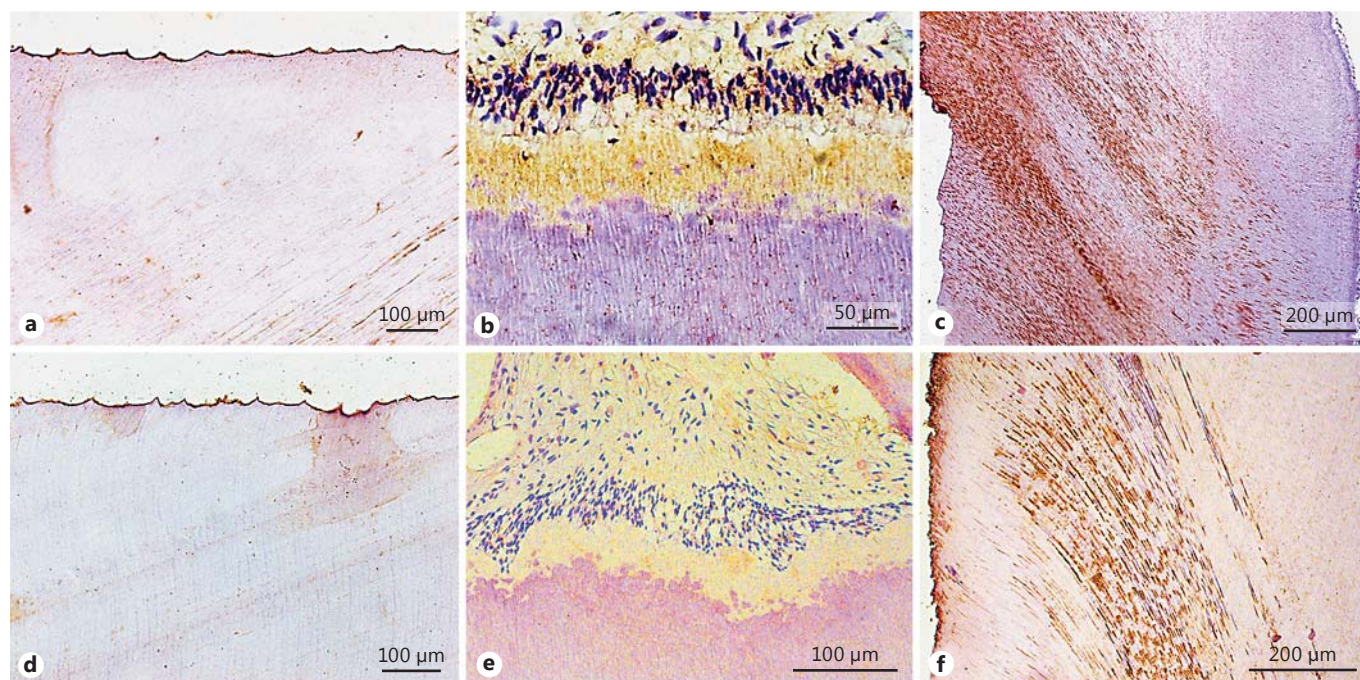


Fig. 3. Immunohistochemical analysis of MMP-20 of the irradiated (group 1; **a–c**) and nonirradiated (group 2; **d–f**) specimens. **a** Expression along the DEJ of an irradiated specimen. **b** MMP-20 expression in predentin, odontoblasts, and pulpal fibroblasts with remarkable dot pattern cytoplasmic staining within the mature odontoblasts (**c**). Intense staining corresponding to the front of the

RRC demineralization in cervical caries and within dilated dentinal tubules towards the pulp (**d**). The same pattern of DEJ positivity in a nonirradiated tooth. **e** Corresponding predentin, odontoblasts, and pulp immunostaining. **f** MMP-20 positivity of superficial caries and peri- and intratubular staining of dilated dentinal tubules.

This interesting pattern of rapid onset and progression of RRC in post-HNRT patients suggests a possible radiation-driven mechanism in the context of the impact of cancer treatment on teeth, which is not yet well understood [Kielbassa et al., 2002; Silva et al., 2009]. Teeth presenting cervical and incisal/occlusal caries, as presented in this study, with partial or even total delamination of the enamel and crown tooth collapse could be seen in post-HNRT patients [Kielbassa et al., 2002]. Even though these seem to be relevant to the clinical characterization of RRC, patients with other severe xerostomia and hyposalivation conditions, such as Sjögren syndrome [Napeñas and Rouleau, 2014] and postallogegenic bone marrow transplantation [Santos-Silva et al., 2015], are susceptible to a similar type of caries. Therefore, this consideration undermines the idea of main direct radiation damage to the dental tissues leading to RRC [Walker et al., 2011] but it does not exclude that it can overlap the hyposalivation and other indirect effects brought on by HNRT [Lieshout and Bots, 2014].

Many authors have tried to correlate this propensity for enamel loss and decreased mechanical properties

with radiogenic damage to dental collagen and DEJ constituents in order to identify the mechanisms responsible for these events [Kielbassa et al., 2002; Fränzel et al., 2006; Springer et al., 2005]. Some have suggested that this feature is due to postradiation instability in the organic components of the DEJ, which could reduce anchoring between enamel and dentin [Pioch et al., 1992; Jansma et al., 1993; Grotz et al., 1997; Kielbassa et al., 2002; Franzel et al., 2006]. In contrast to these assumptions, the present study is the first to reveal that the DEJ of *in vivo* irradiated teeth receiving high doses of radiation retained its normal characteristics of gnarled and smooth patterns without disruptions or enamel-dentin clefts. Furthermore, morphological preservation of the enamel components, including lamellae, spindles, and tufts, which are considered to play an important role in terms of adherence capacity and mechanical strength between the enamel and the dentin, seemed to be unchanged by radiation on optical light microscopy analysis, as were the other expected unchanged enamel and dentin-pulp complex developmental components.

The histologically normal odontoblast morphology, the palisade construction of the odontoblast cell layer, and the unaffected pulp tissue confirm similar findings of previous studies [El-Faramawy et al., 2013; Faria et al., 2014], as well as the formation of reparative tertiary dentin in response to caries that has also been demonstrated previously [Silva et al., 2009]. Together, these findings strongly indicate that in post-HNRT teeth the pulp not only remains vital but also retains its capacity to respond to external irritation, such as caries.

Micromorphological analysis demonstrated that dentin caries presented classical characteristics: an outer layer of fully demineralized and denatured collagen matrix with bacterial colonization (caries-infected layer) and a partially demineralized structurally unaffected organic inner layer (caries-affected layer) [Fusayama, 1997]. Therefore, the current results also support that HNRT does not affect the histological pathways of dentin caries progress in cancer patients [Silva et al., 2009]. This finding also reinforces the contemporary concept that radiation does not impair dentin bond strength, indicating that HNRT patients can be subjected to conventional adhesive restorative procedures [Silva et al., 2010; Galetti et al., 2014].

RT has been directly associated with the regulation and activation of MMP in the brain and many other tissues [Lee et al., 2012]. Recent studies have demonstrated that endogenous dentinal and salivary MMP can play a major role in dentinal caries and erosion pathology [Tjäderhane et al., 1998; van Strijp et al., 2003; Buzalaf et al., 2015; Tjäderhane et al., 2015]. At least MMP-2, MMP-3, MMP-8, MMP-9, and MMP-20 have been indicated to participate in the process of dentin caries [Tjäderhane et al., 1998; Sulkala et al., 2002; Vidal et al., 2014].

A recent study showed via proteomic analysis that MMP-20 was enriched in extracts from *in vitro* irradiated crown teeth. To our knowledge, the present study is the first to evaluate immunohistochemical expression of MMP-20 in post-HNRT teeth, in which overexpression could not be observed. Both the irradiated and the nonirradiated groups had apparently the same pattern of expression and no significant differences were observed in any of the analyzed patterns. However, our study does not necessarily contradict the previous results of Mc-Guire et al. [2014a], because radiation could likely affect MMP-20 activity without changing considerably the total protein amount, which cannot be detected by conventional immunohistochemical techniques. Interestingly, as previously reported by Sulkala et al. [2002], dilated dentinal tubules of caries lesions and odontoblasts were mostly in-

tensive for MMP-20 in our samples, but with no apparent regard to irradiation status. Although there is limited knowledge about the role of MMP-20 in caries progression, its detection did not differ between our analyzed groups and does not seem to explain the particular aggressive behavior of RRC.

In summary, we conclude that the direct effects of radiation do not appear to cause morphological changes in dental tissues or generate radiogenic destruction of its components and that neither MMP-20 expression in DEJ nor in the carious front of demineralization are changed by HNRT. Therefore, any of these events alone seems to be a determinant of the onset and progression of RRC, and the direct effects of radiation may not be regarded as an independent factor to explain this aggressive clinical pattern.

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Disclosure Statement

We declare that there are no conflicts of interests for any of the authors.

Author Contributions

W.G.-S., M.M.R., and A.R.S.-S. conceived and designed the experiments. T.B.B. and A.C.P.-R. obtained the samples. W.G.-S., T.S., and M.M.R. performed the experiments. W.G.-S., G.C.J., and A.R.S.-S. analyzed the data. W.G.-S., M.A.L. and A.R.S.-S. wrote this paper. M.P.M., T.S., M.F.G., and L.T. reviewed this paper.

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